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CHARACTERIZATION OF THE EXTRACELLULAR ENZYME SYSTEMS OF *TRICHODERMA VIRIDE* AH124*

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A mycoparasitic *Trichoderma viride* strain was investigated for the production of extracellular enzymes important in antagonism, by using natural and chromogenic substrates. Some of these enzymes, such as β-1,3-glucanases, and low levels of proteases were produced constitutively. Under inductive conditions, the measurable activities of β-1,3-glucanase, protease and aspecific chitinase increased, while for the proteases and β-1,3-glucanases, the levels depended on both the nitrogen and the carbon source. Gel filtration chromatography revealed at least 4 β-1,3-glucanases, 6 proteases, 2 β-glucosidases and 1 β-1,4-*N*-acetyl-glucosaminidase izoenzyme under inductive conditions.

Keywords: Trichoderma viride – extracellular enzymes

INTRODUCTION

The filamentous fungus *Trichoderma viride* is well known to produce numerous different extracellular enzymes. Some of these enzymes take part in the decomposition of plant litter and are responsible for the survival of the strain in the soil [13], while others play a role in the degradation of fungal cell walls [14]. The quality and amount of these latter enzymes are important as concerns the biofungicidal efficacy of the strain, as they determine its mycoparasitic ability, one of the components of its antagonistic properties. The roles of β-1,3-glucanases, chitinases and proteases in mycoparasitism in *Trichoderma harzianum* have been demonstrated [3].

The aim of this work was to examine the extracellular enzyme systems of *T. viride* AH124, a strain with the ability to antagonize effectively the filamentous fungus *Fusarium culmorum,* which causes extensive damage during the germination of maize plants.

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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MATERIALS AND METHODS

Strains and culture media

T. viride strain AH124 was isolated from the soil of the forest at Ásotthalom, southern Hungary, by Manczinger et al. It was characterized as a strong antagonist against *Fusarium culmorum* (unpublished results). The strain was maintained on minimal medium [11] at 25 °C.

The inductive and non-inductive liquid media for the investigation of enzyme production were as follows: T1 (20 g/l alanine, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T2 (20 g/l alanine, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T3 (5 g/l glucose, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T4 (5 g/l glucose, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7\ H_2O$); T5 (10 g/l glycerol, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T6 (10 g/l glycerol, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T7 (5 g/l mannitol, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T8 (5 g/l mannitol, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \times 7H_2O$); T9F (20 g/l dried *Fusarium culmorum* hyphae). For induction, the T1–T8 media were supplemented with either 20 g/l dried *F. culmorum* hyphae, or 0.5 g/l laminarin, 0.5 g/l colloidal chitin or 0.5 g/l gelatine. Production of *F. culmorum* hyphae: The conidia were inoculated into 100 ml liquid YEGK medium (5 g/l yeast extract, 10 g/l glucose, 5 g/l KH_2PO_4) and incubated in 500 ml Erlenmeyer flasks on a rotary shaker at 180 rpm and 25 °C. After 3 days, the mycelia were collected by filtration, washed with distilled water and dried under vacuum.

Preparation of supernatants for enzyme assays

The conidia of strain AH124 were inoculated into the appropriate inductive or non-inductive liquid media and incubated in 50 ml Erlenmeyer flasks on a rotary shaker at 150 rpm. After culturing at 25 °C, the mycelial pellets were removed by centrifugation (3000 g for 10 min), and the enzyme activities were measured in the supernatants.

Spectrophotometric measurement of extracellular enzyme activities

The mass of hyphae and the protein content of the culture supernatants were determined on the basis of the organically bound phosphorus content [7] and by the method of Spector [16], respectively. For the measurement of aspecific protease, specific trypsin-like and chymotrypsin-like protease activities, azocasein [9], *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide [5] and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide [1] were used, respectively. β-1,3-Glucanase activities were detected by using the dinitrosalicylic acid method [18], with laminarin as substrate. Aspecific chitinase activity was assayed by the method of Rodriguez-Kabana et al. [15]. β-1,4-*N*-acetyl-

glucosaminidase and β-glucosidase activities were determined with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide [20] and *p*-nitrophenyl-β-D-glucopyranoside [8] as substrates, respectively. The substrates (all derived from Sigma) were dissolved in phosphate buffer (pH 6). All experiments were repeated three times, and the measurements were carried out with a Beckman DU-65 spectrophotometer.

Gel filtration chromatography

For gel filtration experiments, strain AH124 was inoculated into 100 ml T9F medium and incubated in 500 ml Erlenmeyer flasks on a rotary shaker at 180 rpm and 25 °C for 5 days. From the vacuum-concentrated supernatant of the centrifuged culture filtrate, 1.5 ml amounts were fractionated on a 0.9×60 cm Sephadex G-100 column (Pharmacia). The column was equilibrated and eluted with 10 mM Tris·HCl buffer (pH 6.8). One ml of the same buffer was added to each collected 0.2 ml fraction. Enzyme activities were determined with a spectrophotometer as described above, but the substrates were dissolved in 10 mM Tris·HCl buffer (pH 6.8) containing 1 mM $MgCl_2$. The mixtures of 0.5 ml of the diluted fractions and 0.5 ml of the substrate solutions were incubated at 30 °C for 2 hours.

RESULTS

Production of extracellular enzymes under non-inductive and inductive conditions in the presence of different carbon and nitrogen sources

The media used for this experiment were the T1-T8 media, both alone and supplemented with *F. culmorum* hyphae as inducer, and also the T9F medium. Aspecific chitinase was produced only under inductive conditions (Table 1). Aspecific protease and β-1,3-glucanase showed low levels of constitutive activity, which in case of β-1,3-glucanase depended on both the carbon and the nitrogen source used: the level of consitutive β-1,3-glucanase activity proved to be the highest in the $NaNO_3$-containing media, with glycerol as carbon source (T6), while in the presence of NH_4Cl (T5) strong repression was detected. Under inductive conditions, the measurable activities of all investigated enzymes increased in every type of culture medium. In most cases, glycerol as carbon, and $NaNO_3$ as nitrogen source promoted the secretion of the enzymes to the highest level.

Production of extracellular enzymes as a function of time under inductive and non-inductive conditions

The activities of aspecific protease, β-1,3-glucanase and β-1,4-*N*-acetyl-glucosaminidase were measured for 8 days and referred to the mass of hyphae. The coni-

Table 1
Secretion of extracellular enzymes under non-inductive (NI) and inductive (I) conditions

Medium	Carbon source	Nitrogen source	Aspecific protease (OD_{360})		β-1,3-Glucanase (OD_{540})		Aspecific chitinase (OD_{585})	
			NI	I	NI	I	NI	I
T1	alanine	NH_4Cl	0.009	0.029	0.000	0.046	0.000	0.134
T3	glucose	NH_4Cl	0.013	ND	ND	ND	0.000	ND
T5	glycerol	NH_4Cl	0.002	0.473	0.000	0.034	0.000	0.248
T7	mannitol	NH_4Cl	0.007	0.092	0.023	0.053	0.000	0.068
T2	alanine	$NaNO_3$	0.010	0.047	0.004	0.036	0.000	0.088
T4	glucose	$NaNO_3$	0.003	ND	ND	ND	0.000	ND
T6	glycerol	$NaNO_3$	0.009	0.608	0.063	0.084	0.000	0.168
T8	mannitol	$NaNO_3$	0.007	0.434	0.015	0.065	0.000	0.195
T9F	*Fusarium* hyphae		ND	0.170	ND	0.032	ND	0.019

ND: not determined

dia were inoculated into T7, T8, and three derivatives of the T5–T8 culture media, containing the appropriate inducers (laminarin, colloidal chitin or gelatine).

Aspecific protease activity was detected in the samples of both the non-inductive and the inductive cultures as early as day 1, but much higher levels appeared under inductive conditions. From the second day, the activities of aspecific proteases decreased in all media, possibly as a result of self-degradation. The level of secreted proteases was higher when $NaNO_3$ was used as sole nitrogen source than that produced in the presence of NH_4Cl. The level of enzyme released into the medium was higher when glycerol was used for incubation than when mannitol was used.

β-1,3-Glucanase activity appeared on the first day of cultivation, even under non-inductive conditions, but this activity was repressed in the presence of NH_4Cl as sole nitrogen source. From the second day, the activity of the initially secreted β-1,3-glucanases gradually decreased under inductive conditions throughout the investigation period, in contrast with the constitutive enzyme level, which showed a slight increase. The level of secreted β-1,3-glucanase was higher when glycerol was used as sole carbon source than that when mannitol was used.

A low level of β-1,4-*N*-acetyl-glucosaminidase activities was detectable on the first day after inoculation. Surprisingly, higher enzyme activities were measured in the samples derived from non-inductive cultures than in those derived from inductive cultures, which was possibly due to the retardation of the growth of hyphae in the presence of chitin. The level of the constitutively secreted enzymes increased until the third day, and then slowly decreased until the end of the investigation period. The level of secreted β-1,4-*N*-acetyl-glucosaminidases was higher in the presence of $NaNO_3$ as sole nitrogen source than that produced in case of NH_4Cl.

Fractionation of enzymes by gel filtration chromatography

Gel filtration detected at least 6 peaks of aspecific protease activity (Fig. 1A), 4 of them in the high, and the others in the low molecular weight fractions. Six peaks of trypsin-like protease activity (Fig. 1B) and 5 peaks of chymotrypsin-like protease activity (Fig. 1C) were detected, one of each in the high molecular weight fractions. The similarity of the profiles of trypsin-like and chymotrypsin-like activities suggests the possibility of the presence of protein-degrading enzymes with both specific activities. Two peaks of β-glucosidase activity were detected in the high molecular weight fractions (Fig. 1D). In addition to 1 peak detected in the high molecular weight fractions, 3 further lower peaks of β-1,3-glucanase activity (Fig. 1E) were detected in the low molecular weight fractions. On the basis of these experiments, 1 peak of β-1,4-*N*-acetyl-glucosaminidase activity could be detected (Fig. 1F).

DISCUSSION

The extracellular enzymes important in mycoparasitism were produced by *T. viride* strain AH124 in large volumes under inductive conditions. With respect to induction, the effects of the presence of either another filamentous fungus, such as *F. culmorum* hyphae, or laminarin, chitin or gelatine were the same. With the exception of aspecific chitinase, the investigated enzymes were produced under non-inductive conditions, but the constitutive levels were low. The activities of β-1,3-glucanase, aspecific chitinase and aspecific protease were repressible by both certain carbon and certain nitrogen sources. In general, the level of secreted enzymes was higher when $NaNO_3$ was used as sole nitrogen source than that produced in the presence of NH_4Cl, and the same effect was observed for the carbon sources glycerol and mannitol, respectively. Under inductive conditions, the levels of constitutive enzyme activities increased, with the exception of β-1,4-*N*-acetyl-glucosaminidase, where the presence of chitin repressed the growth of hyphae. Gel filtration chromatography revealed the complexity of the protease system of *T. viride*: 6 trypsin-like and 5 chymotrypsin-like proteases were detected. Geremia et al. [4] reported on the isolation and purification of an extracellular alkaline protease from *T. harzianum* with a presumed role in mycoparasitism, but there have been few publications on the extracellular proteolytic systems of *Trichoderma* species. The number of extracellular enzymes found in *T. viride* strain AH124 is in conformity with the isoenzyme data in the literature: 2 β-glucosidases were described in *Trichoderma pseudokoningii* [2] and in *T. reesei* [17], 7 β-1,3-glucanases were detected in *T. harzianum* upon induction with laminarin [19], and 2 β-1,4-N-acetyl-glucosaminidases were characterized by Haran et al. in *T. harzianum* [6].

The present study examined the extracellular enzymatic systems of *T. viride* AH124, because previous examinations had indicated that this strain is a promising candidate for biocontrol and, importantly, the methods available for the further breeding of *T. viride* strain AH124 include not only protoplast fusion and mutagenesis, but also transformation based on hygromycin B resistance [12].

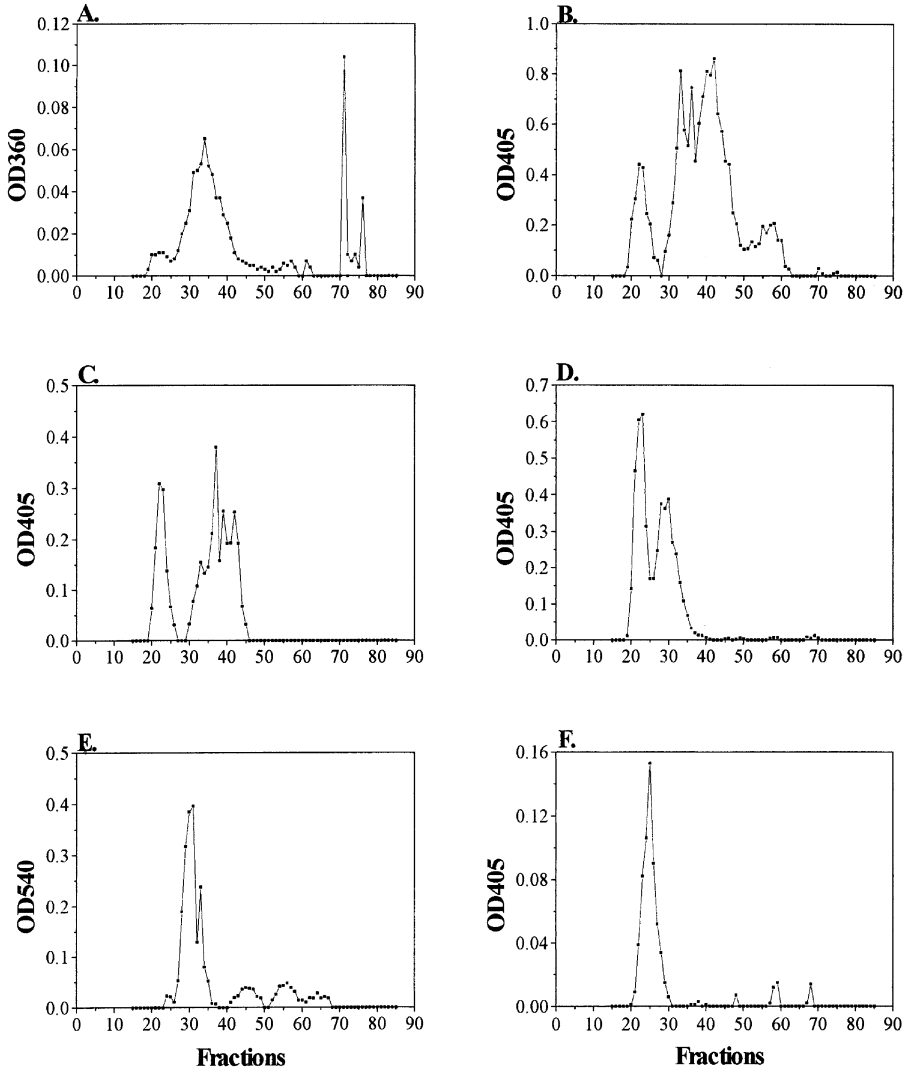

Fig. 1. Extracellular enzyme profiles of *Trichoderma viride* AH124. A. Aspecific protease activity, B. trypsin-like protease activity, C. chymotrypsin-like protease activity, D. β-glucosidase activity, E. β-1,3--glucanase activity, F. β-1,4-*N*-acetyl-glucosaminidase activity

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